Gene Therapy for Retinal Diseases

30

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Abstract

Inherited retinal dystrophies (IRDs) are caused by mutations resulting in progressive functional loss of photoreceptors. The onset of these disorders could be by birth or affect an individual across various ages. Patients diagnosed with Leber congenital amaurosis, retinitis pigmentosa, Stargardt disease, macular dystrophies, choroideremia, etc. experience gradual vision impairment or blindness. Several genes responsible for these dystrophies are known based on extensive genetic studies which led to an understanding of their structure, function, and involvement in cellular pathways making them potential targets for therapeutics. Gene therapy using various delivery vectors such as recombinant adeno-associated virus (rAAV) as a treatment modality offers hope in such conditions that currently have no cure. This chapter provides an overview of different retinal diseases, key genes involved and their mutations resulting in pathological and clinical features, and gene therapy approaches applied. Safety and efficacy are the primary considerations for any gene therapy study. Developments in vector design, promoter modifications, split-gene strategies to express large expression cassettes, compatible vector serotypes or strains to use for efficient retinal cell transduction, alternate gene delivery systems, immune challenges such as the presence of neutralizing antibodies and other toxicity would be given special emphasis in this chapter. Some of the recent success stories of retinal gene therapy preclinical studies and clinical trials are discussed.

Keywords

Inherited retinal dystrophies · rAAV · Gene therapy · AAV serotypes · Retina · Animal models

30.1 Introduction

Over the last several years, the knowledge of genetics and genetic mutations driving various diseases including inherited retinal diseases has grown exponentially. This cumulative knowledge from human and animal model studies raised the hope of genetic therapies (Fig. 30.1). The eye is an easily accessible and fascinating organ for gene therapy. Gene delivery is safer as the eye is highly compartmentalized and has immune privilege to a certain extent. The presence of blood–retina and blood–aqueous barriers limit infection processes or immune activity and thus also hin-

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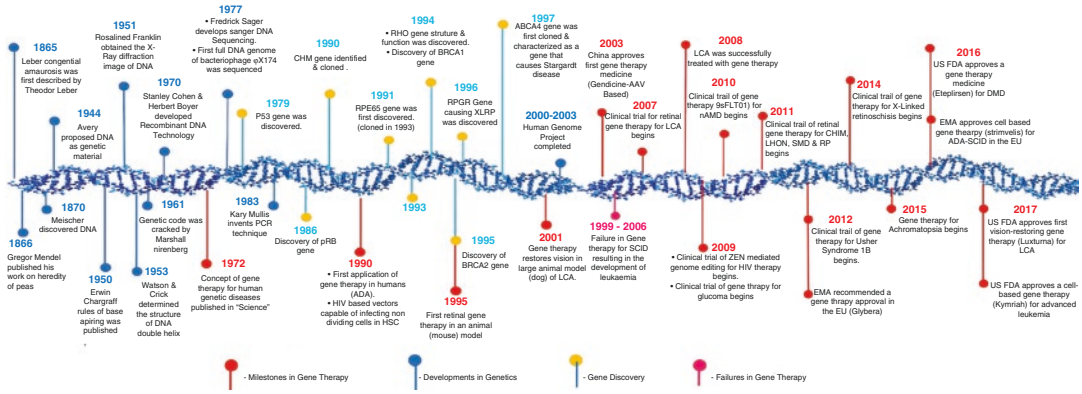

Fig. 30.1 Milestones in retinal gene therapy. The progress in understanding diseases with discoveries in genetics and remarkable technological developments in the field of molecular biology and applications leading to gene therapy products are time lined in this figure

ders gene delivery parenterally to the typical ocular targets such as the retinal pigment epithelium (RPE) and neuronal retina. Thus, gene delivery to the internal layers of the eye depends on ingenuous surgical methods developed over the past few decades such as subretinal injection, supracameral injections, intravitreal injections, etc. Such methods deliver the genes directly to the target tissues while minimizing immune responses outside of the eye. Lack of active intraocular immune responses in the eye also protects vector transduced cells from being lost due to rejection. Most cellular layers in the eye do not replicate and hence a single appropriate dose of viral vector carrying the therapeutic gene is required for efficient transduction and prolonged gene expression. Though recombinant adeno-associated virus (rAAV) has a packaging capacity of around 4.7 kb, it is the vector of choice in clinical trials for ocular gene therapy. Intravitreal and subretinal injections of AAV serotype 1, 2, 5, 7, and 8 have been successfully used for high-level, long-term gene expression in retinal cells [1, 2]. Large genes (example: *ABCA4* and *USH2A*) can be accommodated with improved vector design strategies such as overlapping, trans-splicing, and dual-hybrid vector systems while using rAAV [3]. Other gene delivery methods such as integration deficient lentivirus (IDLVs) to overcome risks of insertional mutagenesis and nanoparticle-based (such as liposomes, polymers, and peptide compacted DNA) have also been studied and successfully used in vivo on retinal cell types. Different nanoparticles have different biochemical properties that govern their internalization, endosomal escape, and transportation to the nucleus. Efficacy of gene transfer can be observed by simple noninvasive procedures like electroretinography and fundus examinations. Availability of both small and large animal models that mimic human disease conditions for several monogenic inherited retinal dystrophies (IRDs) makes it convenient to test strategies for therapeutic benefits in preclinical studies.

Retinal degeneration can be grouped under three broad classifications—(1) hereditary or inherited retinal dystrophies, (2) retinal degenerations, and (3) retinal dystrophies that are part of a syndrome. Some of the most common monogenic IRDs include several forms of retinitis pigmentosa (RP), Leber congenital amaurosis (LCA), Stargardt disease (STGD), choroideremia, achromatopsia, X-linked juvenile retinoschisis (XLRS), Usher syndrome, and other cone–rod and rod–cone dystrophies (CRDs and RCDs). Age-related macular degeneration (AMD), diabetic retinopathy (DR), vein occlusions, and retinopathy of prematurity (ROP) belong to a multifactorial class of degenerations. Similar retinal conditions could be part of syndromes such as Usher, Bardet–Biedlt, and others. Different genes encoding enzymes or structural components contributing to the visual cycle or retinal structure are responsible for various types

of retinal disorders. The retina is a highly specialized structure consisting of light-sensitive cone and rod photoreceptor cells, which initiate neuronal signaling in response to light stimulation. The photoreceptor cells are supported by a monolayer of polarized retinal pigmented epithelium cells (RPE), which performs many key processes including the regeneration of visual pigment that is bleached following light exposure (the visual cycle). Key genes such as retinal pigment epithelium 65 (*RPE65*) retinoid isomerohydrolase, ATP binding cassette subfamily A member 4 (*ABCA4*), MER proto-oncogene tyrosine kinase (*MERTK*), nuclear hormone receptor (*NR2E3*), etc. are responsible for the proper functioning of retinal cells facilitating essential processes such as phototransduction and homeostasis required for normal visual function. The RPE cells are located between the neural retina and the choroid and plays a critical role in the maintenance of visual function. RPE receives light, phagocytoses photoreceptor outer segments, participates in circadian rhythm, is involved in fatty acid metabolism, forms the outer blood–ocular barrier which maintains the subretinal space, performs repair and renewal of cells [4, 5]. RPE cells are polarized and maintain tight cell junctions. Disruption of RPE phagocytosis has been linked to disease phenotypes such as STGD disease and RP [6]. Therefore, the selection of patients for gene therapy requires prior knowledge of the mutated gene as well as the mode of inheritance. Sections in this chapter would include further details on all important considerations for a safer and highly efficacious gene transfer and recent developments in gene therapy, both preclinical and clinical trials for retinal dystrophies.

30.2 Recombinant Adeno-Associated Virus Vectors in Retinal Gene Therapy

Recombinant AAV used in gene therapy contains two inverted terminal repeats (ITRs) retaining the cis genome packaging signal. The *rep* (replication) and *cap* (capsid formation) genes required for virus production are supplied on a trans-helper plasmid. The DNA and promoter of interest placed in between the AAV ITRs is the transgene expression cassette [7, 8]. It can efficiently transduce nondiving cells and is nonpathogenic, which makes it safe for use in gene therapy. There are different strategies to expand the packaging capacity of AAV beyond the traditional 4.7 kb. One such approach is to make a truncated version of a large gene by excluding certain sequences without compromising protein function and retaining functionally relevant sequences [9]. Other strategies such as the cis-activation approach involve dividing the expression cassette into two parts (dual vectors)—one containing the promoter plus enhancer and the other containing the gene are packaged individually. These would get reconstituted upon co-transduction and concatemerization of the ITRs [10]. Additional dual vector approaches include trans-splicing, overlapping, and hybrid strategies. In the trans-splicing approach, the expression cassette is spilt into promoter plus 5′ half of coding sequence and splice donor signals in one vector and the other containing a splice acceptor signal with the 3′ half of the coding sequence. Reconstitution would occur when the 5′ and 3′ vectors form head-to-tail concatamers. Expression is achieved when the intervening double-ITR structure is removed from the mature mRNA using the host cell splicing machinery [11]. Overlapping dual vectors use recombinogenic sequences located in the middle of the gene where the two parts of the gene share an overlap sequence. Upon co-infection with the vectors carrying the two parts, the intact full-length gene is reconstituted by homologous recombination initiated at the overlap to generate the full-length expression cassette, such as alkaline phosphatase (AP) [3]. However, the trans-splicing vector efficiency is dependent on the splice site whereas the overlapping vector efficiency depends on the recombinogenic potential of the overlap sequence. The hybrid dual vector strategy is independent of the transgene properties as it is a novel combination of trans-splicing and overlapping systems [12]. The dual vector strategies may still not meet the need for gene therapy of larger genes (>8.5 kb) such as *CDH23*

known to cause Usher syndrome type ID. A triple AAV system to expand the cargo limits to 14 kb has been tested and shown to be 40% successful compared to single vector systems [13]. In general, the dose of virus, transgene properties, site of injection and serotype used are factors that contribute to an effective gene therapy without side effects of cellular toxicity [14]. A brief compilation of all the preclinical studies (Table 30.1) and AAV based clinical trials (Table 30.2) thus far are provided.

30.2.1 Serotypes for Retinal Cell Transduction

Most IRDs originate from the retinal pigment epithelium cells (RPE) or rod and cone photoreceptors. To choose an AAV serotype that is specific and best for transduction of retinal cell types via any route of administration has been critical. Animal retina, which differ structurally as well (depending on species) to the human retina do not always recapitulate the same surface receptors as human retinal cell types for specific tropism. Ex vivo human retina and retinal pigment epithelium-choroid explants were used to check for transduction efficiency and tropism of AAV2/1, AAV2/2, AAV2/4, AAV2/5, AAV2/6, AAV2/8, and AAV2/9 carrying green fluorescent protein (GFP) driven by cytomegalovirus (CMV) promoter. AAV2/4 and AAV2/5 efficiently transduced photoreceptor cells, the latter being highly specific to the outer nuclear layer (ONL). AAV2/8 exhibited comparatively lower transduction of photoreceptors, whereas higher levels of transduction were observed in the inner retina. AAV2/8 also showed a preference to cone cells in particular. Good transduction of retina is achievable as seen in the pig model injected with AAV2/8 subretinally, suggesting some degree of difference in tropism across species [15]. Interestingly, retinoschisis and CHM gene therapy studies have used the AA8 serotype for gene delivery.

Greater understanding of AAV biology helped design strategies which could overcome proteasome degradation of vectors by incorporating point mutation of surface-exposed tyrosine to phenylalanine (Y-F) in the capsid of rAAV 2, 8, and 9 and achieve greater levels of transduction of retinal cells compared to their wild-type counterparts [16–18]. In a retinal degeneration study, three of the most efficacious AAV capsids AAV2/8(Y733F), AAV2/2(quad Y272, 444, 500, 730F) and AAV2/(7m8) were tested for transduction efficiency in an *rd1* mouse model, monkey and human retinal explants. AAV2/2(7m8) resulted in a greater area of retinal transduction and the highest percentage of gene expression. Transduction of cell types and efficacy of AAV2/2(7m8) and AAV2/2(quad Y-F) via intravitreal or subretinal routes of delivery were similar and better compared to AAV2/8(Y733F). AAV2/2(7m8) was extremely efficient in transducing all retinal cell types compared to the other serotypes which selectively transduced few cell types [19]. rAAV2 (triple Y − F + T − V) efficiently transduced photoreceptors by intravitreal injections [20]. rAAV2/9 and 2/8 transduce RPE, photoreceptors (PR), Muller cells (MC), inner nuclear layer (INL), outer plexiform layer (OPL), and ganglion cells (GC) of mouse and dog retinal cells efficiently and result in high transgene expression [21, 22]. rAAV2/8 and rAAV2/7 are capable of infecting rods and cones at high levels of transduction efficiencies compared to AAV2/5 [23]. Subretinal delivery of rAAV2/5 and rAAV-2/4 carrying CMV. GFP were injected to dog, mouse, and macaque. rAAV2/5 transduced rods and cones better than RPE cells, whereas, with rAAV2/4, transduction was restricted to RPE cells and resulted in long-term gene expression [24]. There has been no report of successful transduction of retinal cells using rAAV2/3 serotype. RPE cells to some extent do get transduced by rAAV2/6 serotype [25]. rAAV/rh10 has been shown to efficiently transduce mice photoreceptor cells and rescue rhodopsin deficient phenotype [26]. rAAVShH10 (close variant of AAV6) has a greater tropism for Muller glial cells as tested in a rat model of RP [27, 28]. A summary of serotypes and their tropism is represented in Table 30.3.

Table 30.1 Preclinical AAV mediated gene therapy studies

AAV mediated retinal gene therapy (Preclinical studies)						
Sl. No.	Human gene	Disorder	Delivery method	Gene therapy for target species	Gene therapy strategy	AAV serotype
1	PDE6B	Autosomal recessive retinitis pigmentosa	Subretinal	Rd1; Rd10 mice	Augmentation; single-stranded oligonucleotide-mediated gene repair	AAV5, AAV8
2	4 Sulfatase	Mucopolysaccharidosis VI	Subretinal	MPS VI Siamese cat	Augmentation	AAV2
3	TYR	Oculocutaneous albinism	Subretinal	Tyrosinase; albino 2 Jackson mice, GPR 143+ mice	Augmentation	AAV
4	ABCA4	Autosomal recessive retinitis pigmentosa, Stargardt 1,cone–rod dystrophy	Subretinal	Abcr^{+} mice	Augmentation	AAV5
5	MYO7A	Usher syndrome 1B (retinitis pigmentosa)	Subretinal	Shaker1 mice	Augmentation	AAV5
6	AIPL1	Retinitis pigmentosa, cone dystrophy, Leber's congenital amaurosis	Subretinal	AIPL1 Hypomorph mouse	Augmentation	AAV8, AAV5, AAV2
7	MERTK	Leber's congenital amaurosis, retinitis pigmentosa	Subretinal	RCS rat	Augmentation	Ad; AAV
8	BBS-4	Bardet-Beidl retinitis pigmentosa	Subretinal	Bbs-4^{+} mice	Augmentation	AAV
9	LRAT	Leber's congenital amaurosis, retinitis pigmentosa	Subretinal	Lrat^{+} mice	Augmentation	AAV
10	CHM	Choroideremia	Subretinal	NAP	Augmentation	AAV2
11	L-Opsin	Red-Green color Blindness (XL)	Subretinal	Squirrel monkey	Augmentation	AAV5
12	CNGA3	Achromatopsia, cone dystrophy	Subretinal	Cnga3^{+} mice	Augmentation	AAV8
13	IMPDH1	Autosomal dominant retinitis pigmentosa 10	Subretinal	Impdh1^{+} mice	Augmentation	AAV
14	CNGB3	Achromatopsia, cone–rod dystrophy	Subretinal	Cngb3^{+}	Augmentation	AAV8
15	CNAT2	Achromatopsia	Subretinal	Gnat2 (Cpfl3) mice	Augmentation	AAV5
16	GUCY2D	Leber's congenital amaurosis	Subretinal	GC1^{+} mice; retinal degeneration chicks	Augmentation	AAV8
17	Whirlin	Usher Syndrome 2D (retinitis pigmentosa)	Subretinal	Whirlin^{+} mouse	Augmentation	AAV2/AAV5
18	RHO	Autosomal dominant retinitis pigmentosa	Subretinal	Rho^{+}, pro23H mouse	Augmentation, zinc finger-based transcriptional repression	AAV
19	RPE65	Leber's congenital amaurosis, retinitis pigmentosa	Subretinal	Rpe65^{+} mice and dog	Augmentation	AAV

Some of the gene therapy studies for candidate genes which are carried out in small and large animal models of various retinal diseases are compiled in this table

Table 30.2 AAV mediated retinal gene therapy clinical trials

AAV mediated retinal gene therapy (Clinical trials)							
Sl. No.	Disease	Human gene	AAV serotype	Delivery method	Trial phase	Sponsor	References
1	Leber's Congenital Amaurosis (LCA 2)	RPE65	AAV2	Subretinal	Phase 3 completed	Spark Therapeutics	A
		RPE65	AAV2	Subretinal	Phase 2 completed	U. College London	B
		RPE65	AAV2	Subretinal	Phase 1 ongoing	U. Pennsylvania, NEI	C
		RPE65	AAV2	Subretinal	Phase 2 ongoing	AGTC	D
2	X-Linked Retinoschisis	RS 1	AAV 2tYF	Intravitreal	Phase 2 ongoing	AGTC	E
		RS 1	AAV 8	Intravitreal	Phase 2 ongoing	NEI	F
3	Choroideremia	CHM	AAV2	Subretinal	Phase 2 completed	U. Oxford (NightstaRx)	G
		CHM	AAV2	Subretinal	Phase 2 ongoing	U. Alberta (NightstaRx)	H
		CHM	AAV2	Subretinal	Phase 2 ongoing	Spark Therapeutics	I
		CHM	AAV2	Subretinal	Phase 2 ongoing	Bascom Palmer (NightstaRx)	J
	Choroideremia	CHM	AAV2	Subretinal	Phase 2 enrolling	U. Tubingen	K
4	LHON	ND4	AAV2	Intravitreal	Phase 2 completed	GenSight Biologics	L
		ND4	AAV2	Intravitreal	Phase 1 ongoing	NEI, Bascom Palmer	M
		ND4	AAV2	Intravitreal	Phase 3 ongoing	GenSight Biologics	N
5	Achromatopsia B3	CNGB3	AAV 2tYF	Subretinal	Phase 2 ongoing	AGTC	O
6	Achromatopsia A3	CNGA3	AAV8	Subretinal	Phase 2 ongoing	U. Tubingen, LMU Munich	P
		CNGA3	AAV 2tYF	Subretinal	Phase 1 enrolling	AGTC	Q
7	Retinitis Pigmentosa	MERTK	AAV	Subretinal	Phase 1 ongoing	King Khaled Eye Specialist Hospital	R
		PDE6B	AAV	Subretinal	Phase 2 ongoing	Horama	S

Reference	Link
A	https://clinicaltrials.gov/ct2/show/NCT00999609
B	https://clinicaltrials.gov/ct2/show/NCT00643747
C	https://clinicaltrials.gov/ct2/show/NCT00481546
D	https://clinicaltrials.gov/ct2/show/NCT00749957
E	https://clinicaltrials.gov/ct2/show/NCT02416622
F	https://clinicaltrials.gov/ct2/show/NCT02317887
G	https://clinicaltrials.gov/ct2/show/NCT01461213
H	https://clinicaltrials.gov/ct2/show/NCT02077361
I	https://clinicaltrials.gov/ct2/show/NCT02341807
J	https://clinicaltrials.gov/ct2/show/NCT02553135
K	https://clinicaltrials.gov/ct2/show/NCT02407678

Table 30.2 (continued)

Reference	Link
L	https://clinicaltrials.gov/ct2/show/NCT02064569
M	https://clinicaltrials.gov/ct2/show/NCT02161380
N	https://clinicaltrials.gov/ct2/show/NCT02652780
O	https://clinicaltrials.gov/ct2/show/NCT02599922
P	https://clinicaltrials.gov/ct2/show/NCT02610582
Q	https://clinicaltrials.gov/ct2/show/NCT02935517
R	https://clinicaltrials.gov/ct2/show/NCT01482195
S	https://clinicaltrials.gov/ct2/show/NCT03328130

Various clinical trials for candidate genes are presented in this table. The sub-table lists the references for the respective trials

Table 30.3 Retinal cell tropism of rAAV serotypes

AAV serotypes	Animal models	Human retinal explants and clinical
rAAV 2/1	RPE	RPE, PR
rAAV 2/2	RPE, PR	RPE, PR
rAAV 2/3	–	–
rAAV 2/4	RPE	RPE, PR
rAAV 2/5	RPE, PR	RPE, PR, ONL
rAAV 5/5	RPE, PR	No data
rAAV 2/6	RPE	RPE
rAAV 2/7	RPE, PR	RPE, PR
rAAV 2/8	RPE, PR, INI, GC	RPE, PR
rAAV 2/9	RPE, PR, INL, GC	RPE, PR
rAAV2/8 (Y733F)	RPE, PR	RPE, PR
rAAV2/2 (quad Y-F)	RPE, PR	RPE, PR
rAAV2/2 (7m8)	All retinal cell types	All retinal cell types
rAAV2/2 (triple Y–F+T–V)	PR	PR
rAAV4/4	RPE	No data
rAAV/rh10	PR	No data

Different serotypes of AAV exhibit selective transduction of the retinal cells, showing varied tropism across species. *RPE:* retinal pigmented epithelium, *PR:* photoreceptors, *INL:* inner nuclear layer, *ONL:* outer nuclear layer, *GC:* ganglion cells
Source: GROW Lab

30.2.2 Broadly Active Versus Specific Promoters

To ensure cell type-specific gene expression, use of a cell-specific or gene-specific promoter is essential to circumvent unwanted transgene expression at off-target areas. An efficient promoter driving high and clinically relevant levels of therapeutic gene expression is necessary so that a single appropriate dose of the vector would be sufficient for treatment. This would overcome consequences of immune response or cellular toxicity resulting from multiple or high virus dosage. Gene therapy studies over the past decades have used broadly active promoters such as CMV [29], human ubiquitin C promoter (UbiC) [30], and chicken beta-actin promoter (CAG) [31]. Some retina-specific promoters used are the RPE-specific promoter—Best1 (bestrophin-1) [32] and RPE65 promoter [33], photoreceptor-specific promoters such as human red opsin (RedO) [34, 35], human rhodopsin (Rho) [23, 35], human rhodopsin kinase (RK) [36], mouse cone arrestin (CAR) [37], etc. Choosing a promoter needs careful assessment as some promoters could pose a certain level of toxicity to the retina such as shortening of the cone outer segment, reduction of the outer nuclear layer, and dysmorphic pigment epithelium [38]. Toxicity due to AAV has been observed in some studies involving animals and humans. Sheep with achromatopsia were treated with AAV2-CNGA3 at a high dose of 10^{12}, showed retinal atrophy and lymphocytic infiltration [39]. Another study, involving NHP eye treated with subretinal AAV8-CNGA3 showed responses of both innate and adaptive immunity [40]. An LCA2 gene therapy clinical trial reported strong evidence of an inflammatory response in five out of eight individuals treated with the higher dose of AAV2-RPE65 [41]. The Alberta choroideremia gene therapy clinical trial, reported

an adverse effect resulting in functional loss of the subject's retina [42]. Broad expression promoters typically may have higher expression levels compared to tissue-specific ones. An example is a study which compared transgene expression by five different promoters—cytomegalovirus immediate-early gene promoter (CMV), human desmin (Des), human alpha-myosin heavy chain (α-MHC), rat myosin light chain 2 (MLC-2), and human cardiac troponin C (cTnC) to drive *LacZ* mediated by AAV9 intravascular delivery in mice. CMV outperformed other tissue-specific promoters resulting in the highest level of transgene expression [43].

30.2.3 Injection Methods

Intravenous injection of AAV would not deliver the therapeutic gene to the target site due to the presence of the blood–retina barrier. Therefore, AAV must be delivered directly to a specific site in the eye where the RPE and photoreceptors are transduced easily (Fig. 30.2). The space between the RPE and photoreceptors, the subretinal space, is the most effective and preferred site for gene therapy. High titer virus preparations are typically required for ocular administration so that the injection volume of the vector may be minimized. In this procedure, a parafoveal bleb is created by subretinal injection of normal saline followed by injection of the vectors through the same self-sealing retinotomy. Preoperative interventions include optical coherence tomography (OCT), pars plana vitrectomy, inner limiting membrane (ILM) staining. Postoperative follow-ups can be done using OCT and fundus scans [44]. During the subretinal injection procedure, there is a risk of reflux of vector into the vitreous which may lead to vitritis due to immune reactions to the capsid.

On the other hand, intravitreal injection is less invasive but may be disadvantageous due to the dilution of the virus within the vitreous or presence of neutralizing antibodies (nAbs) leading to poor transduction of retinal layers [45]. ILM is a wall separating the vitreous and neural retina. A recent injection method called the "subILM," a surgical route to the space between the ILM and neural retina could better serve the purpose of overcoming issues of intravitreal injections [46]. On the basis of AAV being thermostable and negatively charged, yet another recent development was to enhance AAV uptake by applying an in vivo low electric current across the eye to further enhance retinal transduction after intravitreal administration. The electric current was reported to be safe and does not cause damage to the retina structure or function as tested in a wild-type mouse retina using AAV8 [47].

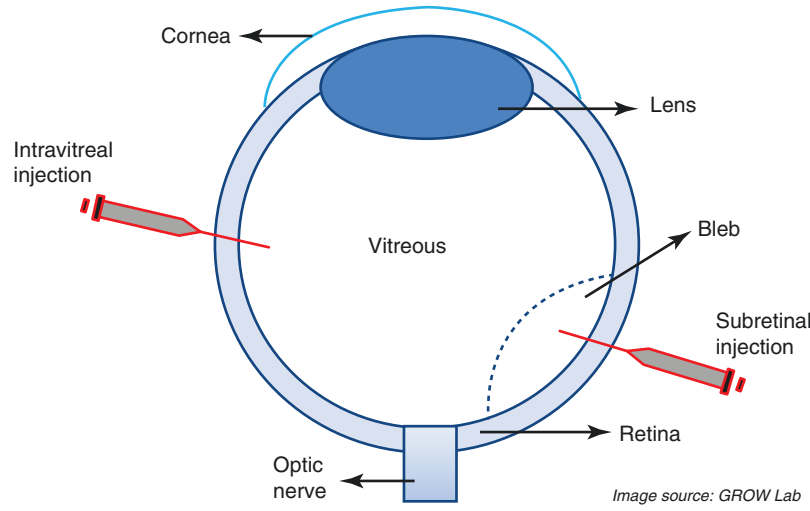

Fig. 30.2 Injection sites for AAV administration. The intravitreal and subretinal spaces are two common sites of injection to deliver therapeutic AAV particles. In the subretinal route, a bleb is created separating the RPE layer and photoreceptors. The intravitreal route is a less invasive procedure where the viral particles are delivered in the vitreous humor

30.2.4 Immune Responses, Biodistribution, and Cellular Toxicity

Recombinant AAVs are known to be nonpathogenic and less toxic. Humans or other animals are naturally exposed to AAV and this poses a challenge to the safety and efficacy of therapy using wild-type AAV capsid. Approximately 80% of the population show the presence of nAbs (neutralizing antibody) to wild-type AAV2 capsid [48–50]. Retina has an immune privilege due to the existence of the blood–retina barriers; therefore, vector administration via the subretinal route has a much reduced risk of negative immune response due to previous exposure to AAV. This is supported by the observation that subretinal readministration of virus led to repeated successful transduction even in the presence of nAbs to AAV in the serum [51, 52].

Post subretinal delivery of AAV2/8 vectors in a canine model, systemic distribution of AAV may occur in other parts such as the brain probably due to trans-synaptic transport to the neurons. This may not necessarily lead to further effects of the transgene if expressed under retinal cell-specific promoters as reported by Stieger et al. [53]. Several years post successful subretinal transfer of AAV 2, 4, and 5, in dogs and primates, existing AAV particles were observed in the outer plexiform layer (OPL) and in all other layers of the retina [54]. Dosage of virus in most of the in vivo gene therapy studies use a range of 10^8–10^{13} vg copies/ml in a single dose. Since the subretinal space can accommodate merely a few microliters, hence a higher viral tier is dosed thereby raising a risk for local tissue reactions, but the currently used injection methods have been shown to be relatively safe with the edema resolving uneventfully in most cases. Other general cellular toxicities arising due to the nature of transgene and transcriptional elements have to be assessed for morphology, physiology and inflammation status in preclinical models before proceeding to clinical trials [38].

30.3 Alternate Methods of Gene Delivery

Apart from AAV, a variety of other vectors have been used to treat retinal diseases which are compiled in Table 30.4. In 1997, lentiviral vectors (LV) carrying GFP driven by CMV or rhodopsin promoter were subretinally injected to the retina of newborn and adult rats and expression followed over 12 weeks. CMV promoter resulted in GFP expression in both photoreceptors and RPE, whereas rhodopsin promoter-driven expression was restricted to the photoreceptors. Due to lack of interphotoreceptor space, expression in adult rat retina was observed only at the site of injection, unlike newborn animals. This long-term expression of the transgenes in photoreceptors could be due to stable integration of the transgene into the genome of the host cell as demonstrated before [55]. The transduction efficiency of LV was similar to adenovirus (Ad)-based vectors [56, 57]. AAV performed better in transducing retina compared to LVs and Ads [58, 59]. Integrase deficient lentiviral vectors (IDLVs) have shown sustained gene expression in vitro and in vivo [60]. These episomal IDLVs are suitable for delivering large genes and transducing nondiving cells in the retina and neural retina without risks of insertional mutagenesis. In 2006, successful use of second-generation self-inactivating (SIN)-IDLVs delivered subretinally in adult rodent models of retinal degeneration ($Rpe65_{rd12/rd12}$ mouse and *Mertk*-deficient rat) showed long-term (9 and 3 months' follow-up in mice and rats, respectively) eGFP expression in adult RPE cells. Thus, IDLVs are potential candidate vectors for gene therapy of retinal dystrophies.

Nanoparticles (NPs) such as polymers, liposomes, peptide compacted DNA have been tested as gene delivery systems for retinal diseases [61, 62]. The advantage of using NPs is the ease in manipulating its chemical properties to suite DNA delivery, low cost of manufacturing, and transferring large vectors without any immune reactions. Critical steps in gene transfer

Table 30.4 Non-AAV mediated retinal gene therapy studies

NON-AAV mediated retinal gene therapy						
Sl. no.	Disease	Gene	Vector	Delivery method	Pre-clinical	Clinical
1	Stargardt disease	ABCA4	Equine Infectious Anemia Virus (EIAV)	Subretinal	$Abcr^{+}$ mice	Phase 2 ongoing https://clinicaltrials.gov/ct2/show/NCT01367444
2	Usher Syndrome 1B	MYO7A	Equine Infectious Anemia Virus (EIAV)	Subretinal	Shaker1 mice	Phase 2 ongoing https://clinicaltrials.gov/ct2/show/NCT01505062
3	Leber's congenital amaurosis	GUCY2D	Lenti virus	Subretinal	$GC1^{+}$ Mice; retinal degeneration chicks	–
4	Leber's congenital amaurosis, retinitis pigmentosa	RPE65	Lenti virus	Subretinal	$Rpe65^{+}$ mice and dog	–
5	Autosomal recessive retinitis pigmentosa	Channel rhodopsin-2	Electroporation	Subretinal	Rd1, Rd10, rd16 mice	–
6	Light damage	Catalase	Adeno virus	Subretinal	Light-damaged mice	–
7	CNV	Retinostat	Equine Infectious Anemia Virus (EIAV)	Subretinal	Laser photocoagulation in mice	Phase 2 ongoing
8	Macular dystrophy	PeripherinRDS	Compacted DNA nanoparticles	Subretinal	Rds^{+}, R172W tg Mouse	–

Lentivirus, equine infectious anemia virus, adenovirus, and nanoparticles are some of the alternate gene delivery methods to treat genetic retinal diseases, some of which are currently in preclinical and clinical trial phases

via NPs involve uptake by target cells, escape endosomal degradation in the cytosol, and transport of genetic cargo to the nucleus without causing cytotoxicity and should be biodegradable in the human body [63]. NPs are taken up by RPE cells by either endocytosis [62]. NPs enter photoreceptors and glial cells by different methods of endocytosis depending on their shape, size, charges, and amount of DNA load they carry [64]. Measures to aid critical processes for efficient gene transfer and expression are considered for prospective human applications. Numerous types, modifications, or customizations of NPs are being studied to make them efficient carriers. The PLGA (poly lactic-co-glycolic acid) and PEG (polyethylene glycol) compacted DNA NPs are the forerunners for safe delivery of genes to photoreceptor cells and the RPE [65]. Cationic liposomes were first used for gene transfer to rat eye via subretinal or intravitreal injections resulting in expression of the lacZ reporter transgene in ganglion cells and RPE alone with no expression observed in photoreceptor cells. This could be due to the phagocytic process of RPE actively taking in most of the NPs compared to less efficient endocytosis by rods and cones [66]. A detailed review of various NPs for ocular gene therapy can be found in Adijanto and Naash's article [64].

30.4 Gene Therapy of Congenital Retinal Degenerations

IRDs are inherited in the family in either autosomal dominant, autosomal recessive, or X-linked manner. The presence of mutational heterogeneity in autosomal dominant conditions is a challenge for gene therapy due to the toxic "gain of function" of the mutant allele. Such conditions lead to the death of photoreceptor cells [67]. The approach to treat a dominant-negative condition typically involves silencing of the dominant allele that is detrimental for the cell, followed by replacement with a codon optimized version of the gene resistant to the silencing. Ribozymes or small interfering RNA (siRNA) are used for silencing the defect. Some common inherited retinal conditions, preclinical studies, and recent clinical trials will be discussed in the following subsections.

30.4.1 Retinitis Pigmentosa (Rod–Cone Dystrophies)

Retinitis pigmentosa (RP) is a progressive rod–cone degeneration (RCDs) caused by mutations in more than 200 genes identified thus far, which affects 1 in 4000 individuals. RP is inherited as autosomal dominant (30–40%) (for example *RHO* gene; 25% of adRP), autosomal recessive (50–60%) (for example, *USH2A* gene; 20% of arRP), X-linked conditions (5–15%) (for example, *RPGR* gene; 70% of XLRP) or some rare forms such as mitochondrial diseases [68, 69]. Mutations in genes responsible for loss of photoreceptors leads to early signs such as difficulty in dark adaptation and night blindness as the rods get affected first. This is followed by a gradual decrease in the visual field (tunnel vision) progressing to complete loss of vision. Electroretinogram (ERG) usually shows a decline in photoreceptor activity in patients with RP. Phenotype and age of onset are highly variable with individuals.

More than 150 mutations in the rhodopsin (*RHO*) gene belong to the G-protein coupled receptor family and present on chromosomal location 3q22.1, which leads to adRP. The structure and function of rhodopsin was described in 1994 [70] The rhodopsin protein is bound to 11-cis retinal (vitamin A) which gets activated upon light stimulation. This event initiates a chain of chemical reactions to produce an electric signal which is sent to the brain and perceived as vision. Gene therapies for *RHO* adRP were either focused on minimizing the expression of the toxic mutant allele or designing a mutation-independent strategy. In a recent gene therapy study, the authors developed a highly efficient shRNA that is specific to human and canine *RHO* in a mutation-independent manner. This vector design also involved the human RHO cDNA, codon-optimized to make it resistant to RNA interference, with both the shRNA and the

RHO gene being expressed from a single AAV virion. This vector was tested in a spontaneously occurring dog model of *RHO*-adRP. The native canine RHO RNA was completely inactivated via subretinal vector delivery leading to expression up to 30% of normal cellular expression. OCT imaging and histopathology of the treated area showed normal structure and presence of normal RHO protein in the remaining transduced photoreceptors. Long-term follow-up of greater than 8 months by OCT and ERG showed reversal of phenotype and a stable maintenance of photoreceptor structure and function. This successful animal model study can be applied to treat patients with this form of adRP by gene therapy [71].

Rare XLRPs are caused by mutations in *RP2* and *OFD1* gene. *RPGR* gene present on chromosomal position: Xp11.4 was identified to cause XLRP in 1996 [72]. This encodes for retinitis pigmentosa GTPase regulator that is necessary for cells' ciliary function aiding vision and is the most common form of XLRP. Along with few naturally occurring [73] and genetically modified mouse models [74], canine models with *RPGR* mutations such as the Siberian husky reported in 1999, which a spontaneous model of XLRP that mimics the human disease [75]. A recently reported model with a deletion of exons 1–4 in *RPGR* gene is the Weimaraner dog, a naturally occurring model for XLRP [76]. *RPGR* gene is a purine-rich gene which is prone to genetic instability, making it difficult to manipulate [77]. In a 2012 preclinical study, rAAV2/5 vector carrying *RPGR* cDNA driven either by a human photoreceptor-specific IRBP (interstitial retinol-binding protein) or GRK1 (rhodopsin kinase) promoter and was delivered at a dosage of 10^{13} vector genome/ml (vg/ml) via the subretinal route. This was not successful as the mice developed toxicity to the mutated therapeutic cDNA [78, 79]. A different report described an RPGR vector strategy where the purine-rich region was deleted in frame resulting in long (deletion of 314 codons) and short (deletion of 126 codons) forms in AAV8.GRK1.RPGR^{ORF15} vectors. The long form showed functional restoration of the photoreceptors in the *Rpgr*-null mouse, whereas the short version did not fare well. This could be due to the maximal glutamylated status of the protein which is required for full therapeutic activity of the protein in the full-length RPGR which may have decreased in proteins formed from reduced sequences [80]. These challenges were overcome by using a codon-optimized version of the gene which is highly stable. AAV. coRPGR^{ORF15} was used to treat *Rpgr*-null and *Rd9* mice mouse models which showed reversal of the phenotype without any toxic side effects, thereby establishing the first successful proof-of-concept leading to three clinical trials initiated in 2017 and 2018 [81]. First clinical trial by Nightstar Therapeutics using AAV8.GRK1.RPGR^{ORF15} (NCT03116113), second by MeiraGTx UK Ltd. using AAV2/5.hRKp.RPGR (NCT03252847), and the third by Applied Genetic Technologies Corp (AGTC) using AAV2/2(YF).GRK1.RPGR^{ORF15} (NCT03316560) [82].

An example of autosomal recessive RP caused by mutations in the *MERTK* gene resulted in the accumulation of outer segment debris due to defective RPE phagocytosis, which is necessary for the renewal of photoreceptor outer segment [83]. In a preclinical study, rats were treated with subretinal injection of AAV-*MERTK* vectors that demonstrated significant improvements in response to ERG [84]. Six patients with confirmed mutations in MERTK were treated by gene therapy in a phase 1 clinical trial. Three out of the six patients had shown improvements in vision and had no signs of systemic toxicity in long-term follow-ups of over 2 years [85]. A 2019 gene therapy phase 2 clinical trial reported final outcomes of six male patients across different age groups affected with choroideremia who had received subfoveal injection of AAV2-REP1 at 10^{11} vg/0.1 mL. These subjects who had documented a reduction in the vision now demonstrated improvements in visual acuity without any adverse toxic effects [86].

30.4.2 Cone–Rod Dystrophies

Gene therapy for retinitis pigmentosa GTPase regulator interacting protein 1 (*RPGRIP1*)- in a canine model of severe cone–rod dystrophy

(CRD) was performed using AAV5 and AAV8. Cone and rod functions were restored and *RPGRIP1* was stably expressed over a period of 2 years in all treated eyes. This large animal model of CRD provides hope toward the treatment of patients [87]. Achromatopsia is caused by mutations in cyclic nucleotide-gated channel alpha 3 (*CNGA3*) (other genes causing similar phenotype are *CNGB3*, *GNAT2*, *PDE6C*, *PDE6H,* and *ATF6*) (http://www.sph.uth.tmc.edu/RetNet/) resulting in poor visual acuity, photophobia, and inability to recognize colors due to cone dysfunctions. The presence of a naturally occurring *CNGB3*-sheep model facilitated the study of treatment strategies [88]. In a 2011 study, sheep deficient in *CNGA3* were treated unilaterally with AAV5 vectors carrying either the mouse or the human *CNGA3* driven by a cone-specific 2.1-Kb red/green opsin promoter [89]. Follow-up studies after 6 years showed the animals had normal vision restored and were measured as demonstrated by ERG and other functional tests. This led to the initiation of clinical trials in *CNGA3* achromatopsia patients (NCT02935517 and NCT02610582) [39].

30.4.3 LCA

Leber Congenital Amaurosis (LCA), first described by Theodore Leber in 1869, encompasses a set of autosomal recessive congenital rod–cone dystrophies (RCDs). The prevalence ranges from 2 to 3 in every 100,000 newborns and is the major cause of blindness in children. Around 15 genes (*IMPDH1, AIPL1, CRB1, CEP290, CRX, GUCY2D, LRAT, RD3, RDH12, MERTK, RPGRIP1, TULP1, SPATA5, RPE65,* and *LCA5*) (http://www.sph.uth.tmc.edu/RetNet/) that are required for normal vision are involved whose mutation result in LCA early in childhood. These genes are responsible for the development of photoreceptor cells, phototransduction and phagocytic processes in normal retina. Other structures such as cilia are needed for the perception of vision. Mutations in *CEP290* (15%), *AIPL1*(12%), *GUCY2D* (12%), and *RPE65* (8%) genes are the most common with other gene mutations accounting for a small percentage (5%) of LCA patients.

Gene therapy for RPE65 in a naturally occurring canine model of RPE65 with visual impairments similar to that observed in human LCA type II were treated with subretinal injection of rAAV-RPE65. The dogs demonstrated improvements in that visual function establishing a proof-of-concept to treat LCA [90]. A long-term follow-up of over 3 years showed stable expression of RPE65 in target areas of the treated eyes and recovered significant retinal function as demonstrated by ERG and other tests [91]. In 2007, the first clinical trial (later two additional trials followed) for LCA had begun with reports of the initial phase of clinical trials in 2008 stating that AAV-RPE65 used were safe and effective over a follow-up of 1 year post treatment in patients [92, 93]. Thereafter, successful Phase I to III clinical trials of gene therapy of RPE65 using Luxturna were reported leading to its approval by the FDA for commercial use in 2017 [94, 95].

30.4.4 Stargardt Disease

Stargardt disease (STGD1) is a juvenile macular degeneration with a prevalence of 1 in 8000–10,000 individuals [96]. However, the age of onset could be during adolescence or any time in adulthood [96]. During the visual cycle, all-trans-retinal is transported out of the discs into the cytosol by ATP binding cassette subfamily A member 4 (ABCA4) transmembrane transporter in the photoreceptors where it gets converted to all-trans-retinol by dehydrogenases (RDH8). Phototransduction results in the formation of bisretinoid A2E (fluorophore of lipofuscin), a by-product that is toxic. The ABCA4 protein is responsible for the removal of these substances out of the cell. A nonfunctional ABCA4 results in the accumulation of these toxic substances causing the death of photoreceptor and atrophy of surrounding RPE cells [97]. Patients with mutations in the *ABCA4* gene experience progressive central vision loss leading to blindness due to death of the photoreceptor cells [98, 99]. Currently, there is no cure for ABCA4 mutation related

to Stargardt disease. ABCA4 cDNA is large (6.8 kb) which would require a suitable vector system such as dual AAVs, IDLVs, or compacted DNA nanoparticles for efficient gene delivery and expression (refer Sect. 30.2 of this chapter). *ABCA4* null mice exhibit phenotypic expression similar to humans [100]. Nanoparticles have been studied in STGD1 mice models where CK30PEG carrying human ABCA4 cDNA and human interphotoreceptor retinoid-binding protein (IRBP) or mouse opsin (MOP) promoters were tested. Expression of ABCA4 was observed at 2 and 8 months post injection and had rescued the phenotype [101]. Traditional AAV vectors are not efficient in the packaging and transduction of large genes [102]. Ghosh lab in India [3] and MacLaren lab in the UK are involved in strategies to package *ABCA4* as split gene into dual AAV vector systems toward treatment for STGD1. Recently, therapeutic levels of *ABCA4* were achieved using the overlapping AAV strategy and which showed the first proof-of-concept in *ABCA4* knockout murine model. Truncated proteins that are formed due to dual vector strategies were reduced by optimizing recombination. Functional ABCA4 protein was observed in photoreceptor outer segments of the mice retina with a successful reversal of the phenotype [103]. This approach could thus be applied to a large animal model followed by clinical trials for gene therapy of Stargardt disease using dual AAVs.

30.4.5 X-Linked Juvenile Retinoschisis

X-linked juvenile retinoschisis (XLRS) is early-onset macular degeneration occurring in males with a prevalence of 1:5000 to 25,000 males worldwide. This condition results from mutations in (*RS1*) gene encoding retinoschisin 1 protein required for cell adhesion, organization, and structural maintenance of the retina. Patients experience poor vision, accompanied by congenital nystagmus, strabismus, vitreous hemorrhage, retinal detachment leading to blindness in severe forms [104]. Preclinical gene therapy studies for retinoschisis were carried out in rabbits and the RS1 knockout mice, where intravitreal administration with self-complementary AAV8-scRS/IRBPhRS showed rescue of the disease phenotype [105, 106]. These successful results led to the initiation of clinical trials and recent reports of phase I/IIa are reported by Cukras et al., [107].

30.5 Gene Therapy of Retinal Neovascularization

Certain retinal pathological conditions like diabetic retinopathy (DR) [108] and age-related macular degeneration (AMD) [109] are caused due to hypertrophic, neovascular formations in the retina and choroid. These are relatively common conditions leading to age-related progressive blindness. AMD affects individuals of age 50 and above. Currently, FDA-approved treatment for these conditions are repeated intravitreal injections of antibodies against VEGF (vascular endothelial growth factor). AAV mediated gene therapy for DR involves strategies to protect nerves and blood vessels from damage or by inhibiting the neovascular networks and vascular hyperpermeability. Antibody approaches targeting sFlt-1, Flt23k, and PEDF have been studied on small and large animal models [110–114]. Other targets to inhibit angiogenesis that are being tested are endostatin, angiostatin, and metalloproteinase-3 [115–117]. Gene therapy to prevent neovascular formations in the case of wet AMD involves AAV2 vectors carrying sFLT-1 and sFLT01 that have been studied in animal models as well as phase 1 and 2 clinical trials. Reports of these studies have demonstrated the treatment approach to be safe and effective in correcting the vision of the majority of patients in clinical trials [118–122].

30.6 Gene Therapy of Syndrome-Associated Retinal Degenerations

Usher syndrome type I is caused by mutations in the myosin VIIa gene (*MYO7A*), present in the RPE. Patients with this syndrome

experience early-onset RP phenotype and hearing loss by birth. A murine model, the shaker1 (*Myo7a*$^{sh1\text{-}4626SB}$) mouse, has been extensively used for preclinical studies [123]. In 2007, the first gene therapy study using third-generation self-inactivating LVs encoding a CMV promoter-driven *MYO7a* gene were administered subretinally in the eyes of the shaker1 mice. The reversal of the phenotype was observed as the ciliary function was restored [124]. A recent study used subretinal injection of an equine infectious anemia virus (EIAV) vector system carrying the *MYO7A* gene driven by CMV promoter, which led to the production of the protein and restoration of vision in knockout mice. Safety was assessed in monkeys which consequently led to the development of UshStat, for clinical trials of Usher type 1B syndrome gene therapy [125].

30.7 Conclusion

The genetic basis of a disease and phenotypic variance were discovered and described by scientists and clinicians from the 1800s (refer Fig. 30.1) There has always been hope to cure or correct the mutations that lead to hereditary disorders. Over the past two decades, gene therapy has developed from ideation to proof-of-concept to clinical trials being conducted across the world. Of all diseases, gene therapy for monogenic inherited diseases, retinal diseases in particular have reached clinical trials early and have now been approved for treatment in many countries. Viral and nonviral methods have been explored for their gene delivery efficiencies to treat various dystrophies. Strategies to overcome and assess adverse immune reactions, toxicities, insertional mutagenesis without compromising gene delivery and expression efficacies are key to a successful gene therapy. Proof-of-concept in vitro and in vivo animal model studies are the initial supportive data that are essential for a treatment approach to achieve clinical approval. With successful clinical reports of patients treated with gene augmentation therapies for retinal dystrophies, "future looks brighter" indeed for other ocular disorders.

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